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Sequence Analysis of SRSV in Fecal Specimens From an Epidemic of Infantile Gastroenteritis, October to December 1995, Japan

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From October to December in 1995, an epidemic of infantile gastroenteritis occurred all over Japan except in Hokkaido and Okinawa prefectures. The number of infected infants and young children was estimated to be over 5 million cases [Editorial, IASR 1996]. The stool specimens from patients were examined for the presence of small round structured viruses (SRSVs) by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing of parts of the RNA-dependent RNA polymerase region. Thirty-five of 87 stool specimens examined gave positive results. Genomic variation was investigated by sequence analysis of a 327 bp cDNA region. The nucleotide and deduced amino acid sequences of the ten strains segregated into two distinct groups; one showed 96.0–100% nucleotide and 99.1–100% amino acid identity, the others showed 91.4–99.7% nucleotide and 93.5–100% identity. The main mechanism of transmission remains unknown. However, these data suggest the possibility of person-to-person spread by two or more kinds of SRSV. ***J. Med. Virol. 52:377–380, 1997.***

KEY WORDS: small round structured viruses (SRSVs); RT-PCR; OTH-25/89/J; sequence

INTRODUCTION

Small round structured viruses (SRSV) are causative agents of airborne, foodborne, and waterborne outbreaks of vomiting and acute gastroenteritis [Kaplan et al., 1982; Morse et al., 1986; Sawyer et al., 1988; Sekine et al., 1989] in children and adults. SRSVs are often transmitted from person to person in hotels, restaurants, and public houses [McDonnell et al., 1995].

Human SRSVs cannot be cultivated in cell or organ cultures or experimental animal models. For a long time since their discovery, the detection of SRSVs depended on electron microscopy (EM) [Caul and Appleton, 1982] and immune electron microscopy (IEM) [Kapikian et al., 1972]. Recently, reverse transcription-polymerase chain reaction (RT-PCR) amplification, cloning, and sequencing methods have been used [De Leon et al., 1992: Jiang et al., 1992]. The SRSVs were classified into two genogroups [genogroup 1 for Norwalk virus-like and genogroup 2 for Snow Mountain agent-like (SMA) and Hawaii agent] on the basis of their sequences in the RNA polymerase region [Ando et al., 1994: Wang et al., 1994].

Infectious gastroenteritis has spread epidemically in Japan in the last quarter of 1995 except in Hokkaido and Okinawa prefectures. SRSV-positive cases were observed from the beginning of October and reached a peak in November [Editorial, IASR, 1996]. Clinical symptoms were characterized by nausea, vomiting and diarrhea and had a median duration of 1 to 2 days.

In this report, we describe the application of RT-PCR, using two primer pairs, and the partial sequence characterization of SRSVs from 5 areas of Japan. Significant genomic diversity has observed.

MATERIALS AND METHODS

Fecal Specimens

The fecal specimens were collected from children with acute gastroenteritis in Japan from October to December 1995. These fecal specimens were free of diarrheagenic bacteria, group A rotaviruses, and adenoviruses.

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A total of 87 fecal specimens, including 11 specimens from Isehara city (Kanagawa prefecture), 33 specimens from Sendai city, 2 specimens from Chiba city, 33 specimens from Kagawa prefecture, and 8 specimens from Sizuoka prefecture were examined by RT-PCR. Isehara, Chiba, and Shizuoka are located in Central Japan, Sendai in Northern Japan, and Kagawa in Western Japan.

RNA Extraction From Fecal Specimens

Viral RNA was extracted from fecal specimens (5–20% suspension in distilled water) by a modification of RNaid method of Ushijima et al. [1992]. Two hundred μl of fecal suspension were added to the same volume of 6 M guanidine thiocyanate in a 1.5 ml microcentrifugation tube. After mixing, 5 μl of RNAMATRIX™ (BIO 101 Inc, LaJolla, Calif.) were added. The samples were mixed and incubated for 1 hour at room temperature on a rocking device. After pelleting 1 min at low centrifugation, the supernatant was removed. The viral RNA bounding RNAMATRIX™ was washed 3 times with RNaid Kit buffer. The samples were centrifugated at 5,000 rpm by microcentrifuge after last wash. The samples were dried and resuspended in 20 μl distilled water. The RNAMATRIX™ bounding SRSV RNA was vortexed until complete suspension. The RNAMATRIX™ suspension was incubated for 10 min at 65°C and centrifuged at 10,000 xg for 2 min. The supernatant containing purified RNA was used for further experiments. The extraction samples from cell culture infected rotavirus were used as a negative control.

Amplification by RT-PCR

The RT-PCR was carried out as a slight modification of the method of Wang et al. [1994]. Two published primer pairs, 35/36 and 81/82, based upon the sequence of the RNA-dependent RNA polymerase region within ORF1 of the Norwalk virus sequence, were used [Wang et al., 1994]. They were expected to produce fragments of 470 bp and 325 bp, respectively. A 5 μl volume of purified viral RNA was heated at 90°C for 5 minutes. After immediate cooling on ice, reverse transcription was performed in a 20 μl reaction volume containing 5 μl of the extracted and purified RNA, 5 units of avian myeloblastosis virus reverse transcriptase (Life Science Inc., St. Petersburg, FL), PCR buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 1.5 mM $MgCl_2$, 1% gelatin], 0.25 μM primer 81 or 35, 200 μM each of dATP, dCTP, dGTP, and dTTP, and 50 units of RNasin. The reaction mixture was incubated at 42°C for 1 hour. After incubation, 30 μl of PCR reaction mixture containing PCR buffer, the second primer 82 or 36, and 1 unit *Taq* polymerase (Boehringer Mannheim GmbH) were added. Amplification consisted of denaturation for 1 minute at 94°C, annealing for 1 min 20 second at 45°C, and extension for 1 minute at 72°C for 40 cycles. The first denaturation time was increased to 3 minutes, and the final extension time was increased to 15 minutes.

For nested PCR, 5 μl of the first PCR products primer pair 35/36 were added to the PCR buffer to make a 50 μl solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 1.5 mM $MgCl_2$, 1% gelatin, 200μM each of dNTPs, 1 μM each of primer 81/82, and 1 unit *Taq* polymerase. Nested PCR was carried out with the same condition of first PCR. The PCR products were analysed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination.

Cloning and Sequencing of RT-PCR Products

Cloning was done using the pCR II vector in the TA cloning Kit (Invitrogen, San Diego, CA), and sequencing was carried out with both strands of the product with the Autocycle™ Sequencing Kit on Automated Laser Fluorescent A.L.F.™ DNA Sequencer II (Pharmacia LKB, Biotechnology, Uppsala, Sweden). The sequences were analyzed using the program A.L.F. Manager (Pharmacia, version 2.1).

RESULTS AND DISCUSSION

National Epidemiological Surveillance of Infectious Agents (NESID) reported that gastroenteritis patients increased from October in 1995, and this phenomenon was earlier than usual. Patients in October to December 1995 greatly outnumbered those for the same period in the previous years. However, detection of rotavirus was seldom reported in the epidemic of this period [Editorial, IASR 1996].

The fecal specimens were collected from hospitalized children from ages 2 months to 11 years in three parts of Japan. Sixty-three percent of patients belonged in the age group 0 to 4 years. A total of 87 fecal specimens were examined by RT-PCR. Using the primer pair 81/82, we found that 35 (40%) of all samples were positive by RT-PCR, but only one (1.1%) sample gave visible product when primer pair 35/36 was used in RT-PCR. Visible products of 327 base pairs (bp) were obtained from 11 (13%) of the 87 fecal specimens examined when the primer pair 35/36 and 81/82 was used in the first and nested PCR, respectively.

The sequence data were obtained by sequencing in both directions using more than 3 clones as a template. We sequenced cloned products made with primer pair 81/82 from 10 specimens obtained from 5 areas (Table I, Fig. 1) and compared the sequence of a 292-base region (excluding the 2 flanking primers 81/82) of the open reading frame 1 (ORF1) with the sequences from the OTH-25/89/J [Wang et al., 1994]. The nucleotide and deduced amino acid sequence identities within this 292 bp interprimer region of 10 SRSV strains are shown in Table II. Clones S-4-1 and S-4-3 derived from the same stool specimens and had different nucleotide sequences. It is possible that this child was infected with two types of epidemic viruses at the same time.

The nucleotide and deduced amino acid sequence of the 10 strains segregated into 2 distinct groups: one consists of 5 SRSV strains, including OTH-25/89/J with 96.0–100% nucleotide and 99.1–100% amino acid intragroup identity; the other consists of the remaining six

TABLE I. Source of SRSVs Yielding Sequences of the Cloned cDNA

	Outbreak		
Clone no.	Year/month	Location	Age group
K-1-1	1995/11	Kagawa prefec.	infant
SY-7-4	1995/11	Sendai city	school child
SS-22-11	1995/11	Sendai city	infant
S-3-2	1995/11	Shizuoka prefec.	infant
S-4-1[a]	1995/12	Shizuoka prefec.	school child
S-4-3[a]	1995/12	Shizuoka prefec.	school child
I-7-5	1995/11	Isehara city	infant
U-8-10	1995/11	Isehara city	infant
K595-1	1995/11	Chiba city	infant
K599-2	1995/11	Chiba city	infant

[a]Clones S-4-1 and S-4-3 were from the same child.

strains, with 91.4–99.7% nucleotide and 93.5–100% intragroup identity (Table II). Differences in nucleotide and amino acid identity between the first and second groups were 18.0–23.9% and 9.3–12.0%, respectively. When the nucleotide sequence of the first group including OTH-25/89/J was compared with that of the second group, deletions were found at positions 4808 in the first and 4820 in the second (Fig. 1). However, each deletion affected only one amino acid sequence (data not shown).

The second 6 strains and UK3/SUMM/88/UK demonstrated considerable nucleotide conservation in the alignment of the nucleotide sequence of the 166 bp fragments (4693–4859) [Ando et al., 1994]. They showed 95.8–97.6% nucleotide and 100% amino acid identity with UK/SUMM/88/UK (data not shown).

The SRSVs were classified into 2 genogroups on the basis of their sequences in the RNA polymerase region [Ando et al., 1994: Wang et al., 1994]. In this study it was demonstrated that SRSVs causing this epidemic are close to SMA-like (including OTH-25/89/J and UK3/SAMM/88/UK) genomically.

Foodborne infections by SRSV have been reported in winter in Japan [Hayashi et al., 1991; Oishi et al., 1992; Sekine et al., 1989; Taniguchi et al., 1979]. OTH-25/89/J-like and SMA/76/US-like viruses were detected as the causative agents in some cases. It seems both viruses were prevalent in many areas of Japan for a couple of decades, but we don't understand how such a big epidemic happened.

One of 87 samples gave positive in the first step RT-PCR using the primer pair 35/36. The primers were able to detect SRSV efficiently, but the annealing temperature was 37°C for the amplification of SMA sample genome [Wang et al., 1994]. In our PCR system, annealing temperature was setting up at 45°C, but not at 37°C, which is one of the reasons for low detection of SRSV by primer pair 35/36. Another possibility was that SRSVs causing this epidemic had considerable mutation at the primer 35/36 region.

SRSVs are an important cause of morbidity due to waterborne, airborne, and foodborne outbreaks of gastrointestinal infection [Kaplan et al., 1982; McDonnell et al., 1995; Sawyer et al., 1988; White et al., 1986]. Person-to-person contact in families is an important mode of transmission. SRSVs in diarrheal stools of infected families can be passed from one family member to another if hygiene or handwashing habits are inadequate. Family members and playmates of these children are at high risk of becoming infected. It is known that SRSV cause food poisoning–like outbreaks, but there has been no report on high incidence of SRSV gastroenteritis in wide area in Japan [Editorial, IASR 1996].

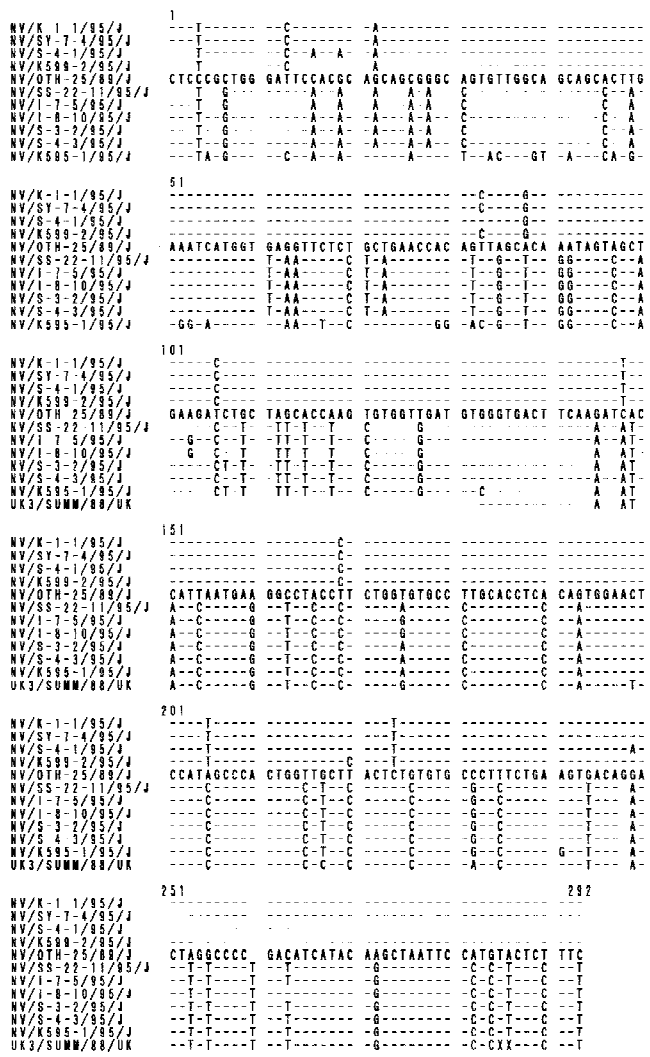

Fig. 1. Alignment of the sequences of ten SRSV clones including the published OTH-25/88/J sequences. The number 1 corresponds to nucleotide 4561 in the genome of Norwalk virus [Jiang et al., 1993]. X represents a residue that could not be determined without ambiguity.

Rotaviruses were the most predominant agents of infantile gastroenteritis in winter. Recently, rotavirus infection tended to shift to early spring in Japan. The peak of rotavirus detection was observed at March and April in the 1995–1996 season (data not shown). This tendency of rotaviruses may be related to large epidemic of SRSV.

However, it is unknown how more than 5 million infants and young children in Japan became infected and ill with acute vomiting and gastroenteritis within about 2 months.

TABLE II. Percentage RNA and Amino Acid Identity Among SRSV Strains

Strain designation	Strain											
	OTH-25	K1-1	SY7-4	S4-1	K599-2	SS22-11	I7-5	I8-10	S3-2	S4-3	K595-1	
OTH-25/89/J		96.6	96.6	96.0	96.2	78.4	78.1	78.4	78.1	78.4	74.0	
K-1-1/95/J	100[a]		100	98.6	99.7	78.8	78.4	78.8	78.4	78.8	75.0	
SY-7-4/95/J	100	100		98.6	99.7	78.8	78.4	78.8	78.4	78.8	75.0	
S-4-1/95/J	99.0	99.0	99.0		98.3	80.1	79.8	80.1	79.8	80.1	75.7	
K599-2/95/J	100	100	100	99.0		79.1	78.8	79.1	78.8	79.1	75.3	R
SS-22-11/95/J	89.7	89.7	89.7	89.7	89.7		99.3	99.3	99.7	100	90.1	N
I-7-5/95/J	89.7	89.7	89.7	89.7	89.7	100		100	99.0	99.3	90.4	A
I-8-10/95/J	89.7	89.7	89.7	89.7	89.7	100	100		99.0	99.3	90.1	
S-3-2/95/J	88.7	88.7	88.7	88.7	88.7	99.0	99.0	99.0		99.7	91.4	
S-4-3/95/J	89.7	89.7	89.7	89.7	89.7	100	100	100	99.0		91.1	
K595-1/95/J	86.6	86.6	86.6	86.6	86.6	93.8	93.8	93.8	92.8	93.8		
	Amino acid											

[a]Percentage identity.

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REFERENCES

Ando T, Mulders MN, Lewis DC, Estes MK, Monroe SS, Glass RI (1994): Comparison of the polymerase region of small round structured virus strains previously classified in three antigen types by solid-phase immune electron microscopy. Archives of Virology 135: 217–226.

Caul EO, Appleton H (1982): The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. Journal of Medical Virology 9:257–265.

De Leon R, Matsui SM, Baric RS, Herrmann JE, Blacklow NR, Greenberg HB, Sobsey MD (1992): Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. Journal of Clinical Microbiology 30: 3151–3157.

Editorial (1996): Viral gastroenteritis, Japan, October to December 1995. Infectious Agents Surveillance Report 192, 17(2):1′ (21′)–2′ (22′).

Haruki K, Seto Y, Murakami T, Kimura T (1991): Pattern of shedding of small, round-structured virus particles in stools of patients of outbreaks of food-poisoning from raw oysters. Microbiology and Immunology 35:83–86.

Jiang X, Wang K, Graham DY, Estes MK (1992): Detection of Norwalk virus in stool by polymerase chain reaction. Journal of Clinical Microbiology 30:2529–2534.

Jiang X, Wang M, Wang K, Estes MK (1993): Sequence and genomic organization of Norwalk virus. Virology 195:51–61.

Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM (1972): Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. Journal of Virology 10:1075–1081.

Kaplan JE, Goodman RA, Schonberger LB, Lippy EC, Gary GW (1982): Gastroenteritis due to Norwalk virus: an outbreak associated with a municipal water system. Journal of Infectious Diseases 146:190–197.

McDonnell RJ, Wall PG, Adak GK, Evans HS, Cowden JM, Caul EO (1995): Outbreaks of infectious intestinal disease associated with person-to-person spread in hotels and restaurants. Communicable Disease Report 5:150–152.

Morse DL, Guzewich JJ, Hanrahan JB, Stricof R, Shayegani M, Deibel R, Grabau JC, Nowak NA, Herrmann JE, Cukor G, Blacklow NR (1986): Widespread outbreaks of clam- and oyster-associated gastroenteritis. Role of Norwalk virus. New England Journal of Medicine 314:678–681.

Oishi I, Tamazaki K, Kimoto T, Minekawa Y (1992): Demonstration of low molecular weight polypeptides associated with small, round-structured viruses by Western immunoblot analysis. Microbiology and Immunology 36:1105–1112.

Sawyer LA, Murphy JJ, Kaplan JE, Pinsky PF, Chacon D, Walmsley S, Schonberger LB, Phillips A, Forward K, Goldman C, Brunton J, Fralick RA, Carter AO, Gary JR WG, Glass RI, Low DE (1988): Twenty-five- to 30-nm virus particle associated with a hospital outbreak of acute gastroenteritis with evidence for airborne transmission. American Journal of Epidemiology 127:1261–1271.

Sekine S, Okada S, Hayashi Y, Ando T, Terayama T, Yabuuchi K, Miki K, Ohashi M (1989): Prevalence of small round structured virus infections in acute gastroenteritis outbreaks in Tokyo. Microbiology and Immunology 33:207–217.

Taniguchi K, Urasawa S, Urasawa T (1979): Virus-like particle, 35–40 nm, associated with an institutional outbreak of acute gastroenteritis in adults. Journal of Clinical Microbiology 10:730–736.

Ushijima H, Koike H, Mukoyama A, Hasegawa A, Nishimura S, Gentsch J (1992): Detection and serotyping of rotaviruses in stool specimens by using reverse transcription and polymerase chain reaction amplification. Journal of Medical Virology 38:292–297.

Viral Gastroenteritis Subcommittee of the Public Health Laboratory Service Virology Committee (1993): Outbreaks of gastroenteritis associated with SRSVs. PHLS Microbiology Digest 10:2–8.

Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, Estes MK (1994): Sequence diversity of small, round-structured viruses in the Norwalk virus group. Journal of Virology 68:5982–5990.